Coastal bacterioplankton community response to diatom-derived polysaccharide microgels.

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Originality-Significance Statement

We have performed a novel experiment to determine bacterioplankton community response to the addition of diatom-derived polysaccharide microgels, including assessing uptake of ^{13}C-labelled microgels using RNA Stable Isotope Probing. A key finding from this study is that the copiotrophic Alteromonadales (genus *Alteromonas*) utilise and assimilate natural diatom-derived polysaccharide microgels (transparent exopolymer particles, TEP).

Summary

Phytoplankton-derived polysaccharide microgels, including transparent exopolymer particles (TEP), are a major component of the marine organic carbon pool. Previous studies have made correlative links between phytoplankton material and bacterioplankton, and performed experiments that assess general responses to phytoplankton, yet there is a lack of direct empirical evidence of specific bacterioplankton responses to natural phytoplankton polysaccharide microgels. In this study, we used diatom produced TEP in controlled incubation experiments to determine the impact of polysaccharide microgels on a coastal bacterioplankton community. Quantification of bacterial 16S rRNA gene transcripts showed that the addition of TEP caused an increase in bacterioplankton activity. Similarly, high-throughput sequencing of RT-PCR amplified bacterial 16S rRNA gene transcripts showed that active bacterioplankton community structure and diversity also changed in response to microgels. Alteromonadales and Rhodobacterales increased in abundance in response to TEP, suggesting that both bacterioplankton taxa utilise diatom-derived microgels. However, through assessing ^{13}C-labelled TEP uptake via RNA Stable Isotope Probing, we show that only the Alteromonadales (genus *Alteromonas*) assimilated the TEP carbon. This study adds utilisation of diatom-derived TEP to the metabolic repertoire of the archetypal copiotrophic bacterioplankton *Alteromonas*, and indicates that the Rhodobacterales may utilise TEP for other purposes (e.g. attachment sites).

Introduction

Transparent exopolymer particles (TEP) are polysaccharide microgels that are primarily formed from the abiotic coagulation of dissolved precursor molecules and constitute up to 40% of total particulate organic carbon (POC), therefore representing a significant component of the marine carbon budget (Passow, 2002; Engel et al., 2014). Phytoplankton are the main source of TEP precursors that are excreted during regular metabolic processes. High precursor excretion rates have been shown particularly in diatoms (Passow, 2002). Detailed chemical analyses of TEP and other marine microgels remains to be conducted, however there is evidence that TEP are heterologous sulfated polysaccharides enriched with fucose, rhamnose and arabinose (Passow, 2002).

TEP and other marine microgels have surface-active properties that make them 'sticky' and consequently act as a glue matrix that holds larger particles together to form aggregates, including marine snow, and therefore have a significant role in the biological carbon pump (Engel et al., 2004). TEP can be neutrally buoyant, becoming enriched in the sea surface microlayer (Azetsu-Scott and Passow, 2004; Cunliffe et al., 2009) and subsequently expelled into the atmosphere via bubble-bursting and wave breaking to form cloud condensation nuclei (Quinn and Bates, 2011). TEP are also utilised as a food source by some zooplankton (Passow and Alldredge, 1999), even in the presence of alternative resources such as diatoms (Ling and Alldredge, 2003). Several previous studies have shown that bacterioplankton attach to microgels, including TEP (Mari and Kiørboe, 1996; Simon et al., 2002; Mari et al., 2004). However, there is currently no direct experimental evidence that identifies bacterioplankton that actively respond specifically to diatom-derived TEP and assimilate TEP carbon.

Bacterioplankton at the Western English Channel time-series site Station L4 (Supplementary Figure 1) have been relatively well characterised, and shown to have annually repeating and seasonal scale patterns of diversity that are linked to changes in phytoplankton production (Gilbert et al., 2012; Taylor et al., 2014). Changes in Station L4 bacterioplankton diversity assessed using 16S rRNA gene high-throughput sequencing

3

(HTS) and co-occurring TEP concentrations during the spring-summer transition showed that there is a correlation between the increase in abundance of the orders Rhodobacterales and Flavobacteriales and the decline in seawater TEP concentrations, suggesting that both taxa could be utilising TEP and therefore have a role in TEP cycling (Taylor et al., 2014). Knowledge of the identity of active bacterioplankton that degrade and assimilate TEP carbon is vital to develop a complete understanding of microgel biogeochemistry.

Mari and Dam (2004) developed a technique to enrich and isolate TEP from seawater using paramagnetic beads that are trapped in microgels as they form from dissolved precursors under abiotic conditions. During this procedure, TEP are formed by bubbling, which mimics natural abiotic TEP formation via bubble scavenging in seawater (Mopper et al., 1995; Zhou et al., 1998). In this study, we applied the approach developed by Mari and Dam (2014) to identify active bacterioplankton in surface coastal waters that respond to and assimilate diatom-derived TEP. To achieve this, we conducted a short-term incubation experiment that assessed active (RNA-based) Station L4 bacterioplankton community responses to the addition of diatom-produced TEP, including determining TEP carbon assimilation via RNA Stable Isotope Probing (SIP) using ^{13}C-labelled diatom-produced TEP.

To identify an ecological-relevant diatom to use as a TEP producer, we assessed time-series data of phytoplankton diversity and abundance at Station L4 collected from 2008 to 2013 (Widdicombe et al., 2010). Diatoms are a dominant phytoplankton group at Station L4, with *Chaetoceros* spp. being a major diatom group (Supplementary Figure 2). We subsequently selected *Chaetoceros compressus* PLY550 from the MBA Phytoplankton Culture Collection as a model TEP producer because it was originally isolated from the Western English Channel. To produce ^{13}C-labelled cultures, 5 mM NaH^{13}CO$_3$ (99.8% ^{13}C label, CK Gas Products) was added to f/2 artificial seawater media (Guillard and Ryther, 1967) as described previously (Taylor et al., 2013). 5 mM NaHCO$_3$ (unlabelled) was added to the ^{12}C control culture flasks. ^{13}C-labelled and ^{12}C control TEP were produced from late exponential phase axenic cultures using established methods (Mari and Dam, 2004). A total

4

of ~1.5 mg TEP carbon was produced for each treatment, with no detectable protein or nucleic acids (DNA and RNA). Surface seawater (2m depth) was collected from Station L4 (27/05/2014; Supplementary Figure 1), filtered through 100 μM mesh to remove large grazing zooplankton and 2 L transferred into replicate incubation vessels. Three incubations treatments were setup; no TEP addition (n = 3), ^{12}C-TEP addition (n = 3) and ^{13}C-labelled TEP addition (n = 3). After TEP addition, the TEP concentration was 900 μg carbon L^{-1} and therefore at an environmentally relevant concentration based on previous sampling at Station L4 (Taylor et al. 2014). The vessels were maintained at 13°C (*in situ* temperature), in the dark and gently aerated continually. After 18 hours, 1 L seawater samples were removed, filtered through 0.2 μm filters and the filters stored at -80°C. RNA was extracted from the filters as previously described (Taylor and Cunliffe, 2015) and cDNA generation was performed using the Omniscript RT kit (Qiagen) in accordance with manufacturer's instructions using the reverse primer PROK1492R (Suzuki et al. 2000). The V4 region of the 16S rRNA gene was amplified by PCR using primers 515F and 806R (Caporaso et al., 2011) from the cDNA and sequenced on an Ion Torrent PGM (Life Technologies) in accordance with manufacturer's instructions. Sequences were analysed using the QIIME software package and the Greengenes reference database as previously described (Taylor et al., 2014), and are available via the European Sequence Archive under the accession PRJEB8281. Q-PCR primers BACT1369F and PROK1492R (Suzuki et al., 2000) were used with the Sensi-FAST SYBR Q-PCR kit (Bioline) and a Qiagen Rotor Gene 3000 (Qiagen) also as previously described (Taylor and Cunliffe, 2015). RNA SIP, including density gradient centrifugation, fractionation and fraction analysis was performed using established protocols (Whiteley et al., 2007; Taylor et al., 2013). RNA from the replicate TEP addition treatments was pooled, resuspended in ultra-pure nuclease free water and added to cesium trifluoroacetate (CsTFA) gradients. After centrifugation, twelve fractions were assessed by quantification of buoyant densities and DGGE fingerprinting of RT-PCR amplified 16S rRNA transcripts and 'heavy' gradient fractions from both ^{12}C control and ^{13}C-labelled incubations were selected for analysis using high-throughput sequencing as described above.

Results and Discussion

Bacterioplankton activity was quantified using 16S rRNA RT-Q-PCR analysis of RNA samples and showed that, compared to the start of the experiment (T0), bacterioplankton activity significantly increased in all microcosms after 18 hours (t-test; p ≤0.04) (Figure 1A). However, bacterioplankton activity was much greater in the microcosms with TEP added compared to the no TEP control incubations after 18 hours (t-test; p 0.04). There was no difference between bacterioplankton activities in the microcosms containing ^{12}C-labelled TEP and ^{13}C-labelled TEP. Active bacterioplankton diversity was determined by high-throughput sequencing of 16S rRNA transcripts amplified by RT-PCR from RNA samples, and showed that the communities in the different microcosms formed distinct clusters (Figure 1B). PERMANOVA analysis of the distribution of the bacterioplankton 16S rRNA-derived operational taxonomic units (OTUs) showed that the communities in the TEP addition microcosms were significantly different to the communities in the no TEP control incubations (PERMANOVA; pseudo-F 23.81; p 0.04). There was no significant difference between bacterioplankton communities in the microcosms containing ^{12}C-labelled TEP and ^{13}C-labelled TEP (PERMANOVA; pseudo-F 2.38; p 0.20).

Further analysis of the bacterioplankton OTUs showed that species richness, determined as the number of OTUs in a fixed sample size of 9,000 randomly chosen sequences (see supplementary methods), significantly decreased in all of the microcosms after 18 hours incubation (ANOVA; p <0.01), and that there was no significant difference between the control microcosms and the microcosms with TEP added (Figure 1C). Species evenness, determined as the calculated Shannon Index also in a fixed sample size of 9,000 sequences, was significantly decreased in the microcosms with TEP added after 18 hours compared to the start of the experiment (ANOVA; p ≤0.02), however there was no significant difference between the control microcosms after 18 hours and T0 (Figure 1D). Again, there was no significant difference between bacterioplankton species richness and evenness in the microcosms containing ^{12}C-labelled TEP and ^{13}C-labelled TEP.

6

At the start of the experiment (T0), the active bacterioplankton community was dominated by the orders Flavobacteriales, Rhodobacterales, Rickettsiales, Alteromonadales, Oceanospirillales and SAR406 (Figure 2A). After 18 hours incubation in both the control microcosms (i.e. no TEP) and the microcosms with TEP added, the order Rhodobacterales significantly increased in relative abundance, but with abundance greatest in the presence of TEP (ANOVA; p ≤0.04). Similarly, after 18 hours in the microcosms with TEP added, the relative abundance of the order Alteromonadales also increased substantially (ANOVA; p <0.01), however, unlike Rhodobacterales, the abundance of Alteromonadales was not increased in the control microcosms after 18 hours. Focusing on the orders Rhodobacterales, Flavobacteriales and Alteromonadales showed that two specific groups were increased in relative abundance in the microcosms containing TEP after 18 hours (Figure 2Bi). From the Rhodobacterales family Rhodobacteraceae, OTUs that were most closely associated to the genera *Roseovarius* and *Amylibacter* (19% and 10% respectively) were significantly increased (ANOVA; p <0.001), as well as OTUs assigned to the Alteromonadales genus *Alteromonas* (ANOVA; p <0.001).

Comparison of 16S rRNA libraries generated from ^{13}C-labelled and ^{12}C control RNA gradient fractions showed that after 18 hours, the genus *Alteromonas* was also enriched in the ^{13}C incubation fraction compared to the ^{12}C control incubation (Figure 2Bii), indicating that the taxa had assimilated the ^{13}C-TEP. However, the Rhodobacterales OTUs were not enriched in the ^{13}C incubation fractions compared to the ^{12}C control incubations, indicating that the group had not assimilated the TEP carbon at that stage.

Alteromonas are copiotrophic bacterioplankton that have already been linked to DOC processing, including the direct uptake of *Chaetoceros*-derived DOC (Sarmento and Gasol, 2012). Transcriptome analysis of microcosms amended with DOC showed that *Alteromonas* responded rapidly to the substrate addition (McCarren et al., 2010), and an *Alteromonas* strain isolated off the Californian coast consumed the entire labile DOC pool in seawater samples (Pedler et al., 2014). Incubation of seawater collected from the Patagonian continental shelf and Mauritanian upwelling showed that the addition of commercially

7

produced polysaccharides stimulated the growth of Alteromonadaceae bacterioplankton that were identified as predominantly related to *Alteromonas* (Wietz et al., 2015).

Alteromonas stimulated by TEP (Figure 2Bi) and that had assimilated TEP carbon (Figure 2 Bii) showed 100% sequence similarity to *Alteromonas macleodii* (KX519548). *A. macleodii* strain 83-1 was isolated from a seawater microcosm amended with the macroalgal cell wall polysaccharide alginate (β-D-mannuronate and α-L-guluronate arranged in alternating homo- and hetero-polymeric blocks) (Neumann et al., 2015). *A. macleodii* 83-1 is able to utilise alginate as a sole carbon source (Neumann et al., 2015) and colonise artificially-produced alginate microspheres (Mitulla et al., 2016). Genome analysis of *A. macleodii* 83-1 and other *A. macleodii* strains has revealed a suite of carbohydrate-active enzyme (CAZyme) encoding genes, including polysaccharide lyases and associated sugar transporters that underpin alginate degradation (Neumann et al., 2015). Extracellular polysaccharides produced by *Chaetoceros* spp. include fucose, rhamnose and arabinose (Gügi et al., 2015), and therefore have a similar composition to TEP (Passow, 2002) and are distinct from macroalgal-derived alginate. However, TEP degrading and assimilating *Alteromonas* bacterioplankton probably utilise similar CAZyme-based systems that are yet to be characterised.

Some studies have linked increased abundance of bacterioplankton Alteromonadaceae, including the genus *Alteromonas,* with experimental approaches that 'confine' seawater, such as bottle incubations and mesocosms (Eilers et al., 2000; Schäfer et al., 2001). However, we show here that *Alteromonas* respond specifically to diatom-derived TEP and assimilate diatom TEP carbon, with no evidence of stimulation in the no TEP controls.

In a previous study at Station L4 we showed a correlation between increased Rhodobacterales and Flavobacteriales abundance and TEP decline over the spring-summer transition, suggesting that both orders utilise TEP (Taylor et al., 2014). Even though Alteromonadales are a major component of the Station L4 bacterioplankton community, especially post-spring bloom, we did not observe any correlation with TEP. This is possibly

8

because of the different time-scales of the two studies and the dynamic nature of coastal bacterioplankton communities; the *in situ* correlative study was conducted at weekly intervals (Taylor et al., 2014) and this study showed TEP carbon assimilation after 18 hours. The rapid response of *Alteromonas* to TEP addition may have been missed in the weekly survey at Station L4. Similarly, Flavobacteriales response to TEP may occur at broader time-scales than analysed here. Also, in this study we used 'fresh' TEP from one diatom species. In natural systems, TEP will be heterologous because it is formed from different taxa and in different chemical states (e.g. aged). Further work is needed to examine TEP from other phytoplankton sources and at different states of decay.

In this study, even though Rhodobacterales activity increased in the presence of TEP, corroborating our previous *in situ* study (Taylor et al., 2014), we did not see any evidence of TEP assimilation after 18 hours. Rhodobacterales may not have the metabolic capability to directly utilise phytoplankton-derived TEP as growth substrates, instead syntrophically depending on primary degraders, such as *Alteromonas*, to produce low molecular weight sugar substrates (Teeling et al., 2012; Buchan et al., 2014; Taylor et al., 2014). Genomic and metagenomic analysis of Rhodobacterales, including members of the Marine Roseobacter Clade, shows a prevalence of genes encoding systems to transfer sugars into the cell (e.g. ABC-type transporters) (Teeling et al., 2012; Buchan et al., 2014).

TEP are readily colonised by marine bacterioplankton (Mari and Kiørboe, 1996; Simon et al., 2002; Mari et al., 2004), and other studies have shown that the Rhodobacterales are important primary surface colonisers in coastal waters (Dang et al., 2008). The increase in Rhodobacterales activity in the presence of TEP could be because of stimulated attached growth. Another possibility is that the Rhodobacterales were stimulated by elements other than TEP carbon. Rhodobacterales are well characterised as being able to utilise organic sulfur compounds, and there is growing evidence they may directly utilise sulfate-containing compounds produced by diatoms (Durham et al., 2015). TEP produced by diatoms have also been shown to contain amino-sugars (Chiovitti et al., 2003), although in relatively low abundance, these could be used a potential source of nitrogen.

Alteromonadales, Rhodobacterales and Flavobacteriales are regarded as 'master recyclers', with growing evidence that they utilise a suite of enzymes to directly and indirectly exploit algal-derived polysaccharides (Teeling et al., 2012; Buchan et al., 2014; Taylor et al., 2014; Neumann et al., 2015; Teeling et al., 2016). In this study, we present experimental evidence to progress this paradigm by demonstrating that the Alteromonadales (*Alteromonas*) respond to diatom-derived TEP and assimilate TEP carbon, and that the Rhodobacterales increase in activity in response to the same biogenic substrate. Further work is needed to establish if members of these groups are generalists or specialists for specific algal-derived polysaccharides (e.g. macroalgal vs. microalgal), and to determine if and how these groups interact to breakdown and re-cycle these compounds via ecological processes such as syntrophy. This work should include determining the underpinning metabolic pathways involved in TEP assimilation and TEP-associated syntrophy. Given the dynamic nature of coastal marine plankton communities, future work should also consider high-resolution time-series sampling and studying TEP from a broader range of representative phytoplankton.

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Figure Legends

Figure 1. (A) Abundance of 16S rRNA transcripts determined by quantitative reverse transcription PCR (Q-RT-PCR) analysis of RNA isolated from seawater samples. Values shown are means ± standard deviation (n = 3). (B) Principal coordinates analyses plot describing diversity of active bacterioplankton communities determined from 16S rRNA libraries using UniFrac distance matrices generated from OTU (97% similarity) data. The green circles represent the ^{12}C-TEP incubations and the green triangles represent the ^{13}C-TEP incubations. (C) Species Richness determined as the number of OTUs, and (D) Species Evenness determined as the calculated Shannon Index, in a fixed sample size of 9,000 sequences. Values shown are means ± standard deviation (n = 3).

Figure 2. (A) Normalised abundance of major bacterioplankton orders, and (Bi) specific taxa within the orders Flavobacteriales, Rhodobacterales and Alteromonadales in the 16S rRNA libraries. Values shown are means ± standard deviation (n = 3). (Bii) Comparison of the normalised relative abundance of 16S rRNA gene sequences from ^{13}C-labelled fraction and ^{12}C control fraction. Taxa >0 (i.e. *Alteromonas*) are those taxa enriched in the ^{13}C libraries relative to the ^{12}C control libraries, indicating that they had assimilated ^{13}C-labelled TEP.
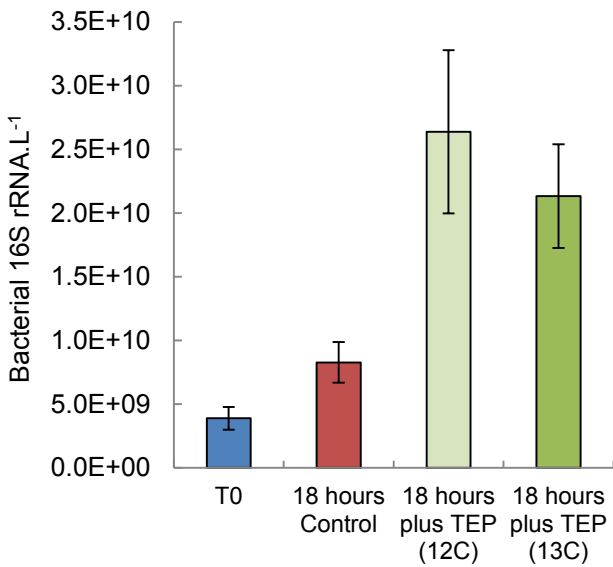
Supplementary Figure Legends

Supplementary Figure 1. Map showing the location of Station L4, which is part of the Western Channel Observatory in the Western English Channel.
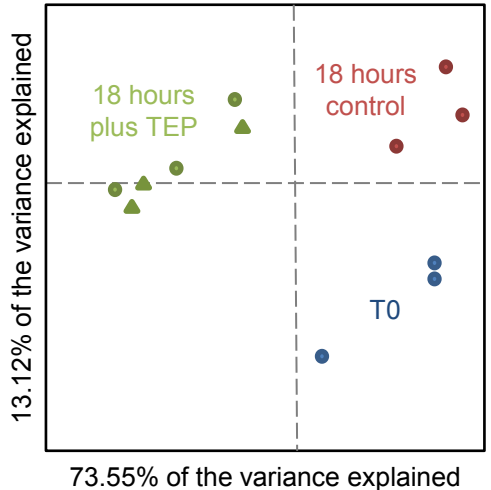
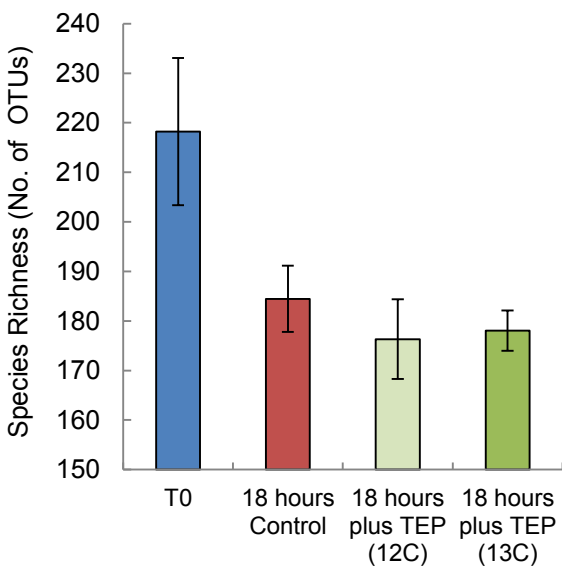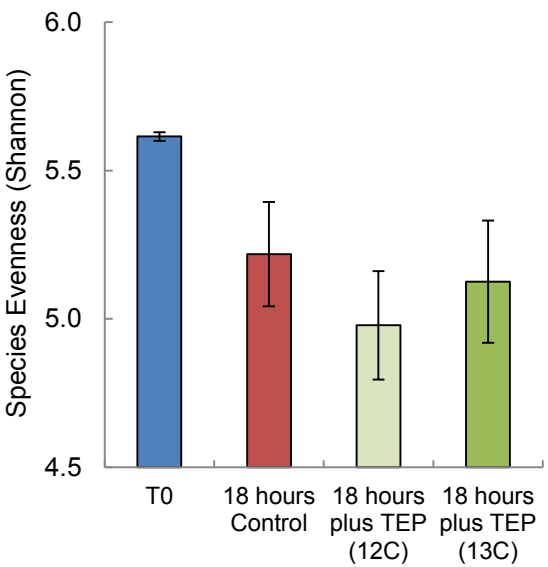Supplementary Figure 2. Abundance of diatoms and other phytoplankton at Station L4 from 2008 to 2013.

A.

B.

C.

D.

Figure 1

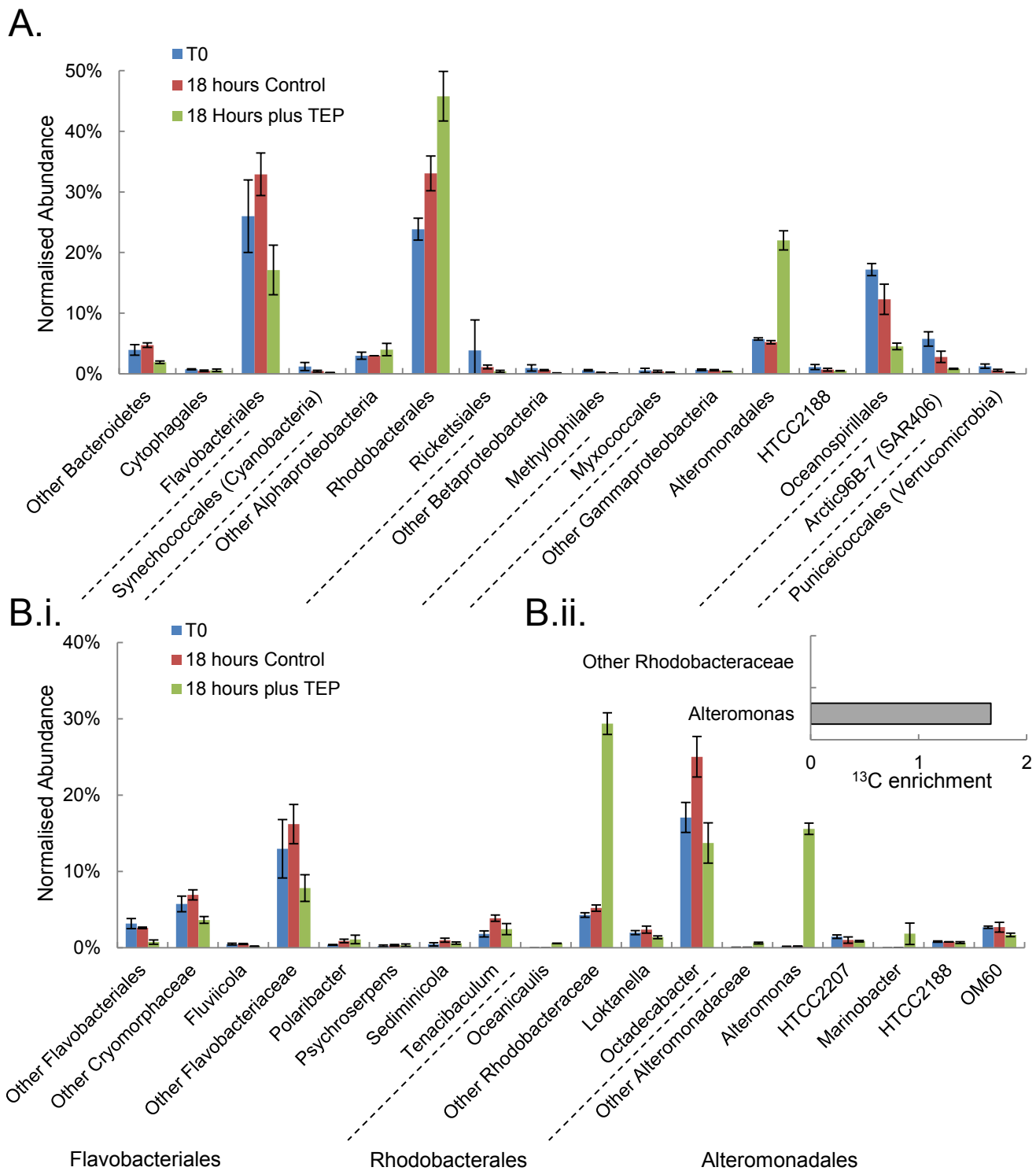A.

B.i.

B.ii.

Figure 2